Capillary Electrophoresis Detection

See  II/ELECTROPHORESIS / Detectors for Capillary Electrophoresis

Capillary Electrophoresis–Mass Spectrometry

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Introduction

Capillary zone electrophoresis (CZE) is widely recognized as a powerful analytical technique in its own right, known for its high separation efficiency, short analysis times and low-volume sample requirements. These characteristics made CZE a popular method for the analysis of peptide mixtures, protein digests, drug substances and biotechnological products. The coupling of CZE with electrospray ionization mass spectrometry (ESI-MS), first reported by Olivares et al. in 1887, has added further capabilities, in particular for obtaining molecular mass information and structural details when tandem mass spectrometry (MS-MS) is used. However, it can be said that the major advantage of such coupling is that the migration time is not the only parameter used for identifying the eluted components. These times are subjected to variations between runs, yet such variations become irrelevant when, in the same run, highly diagnostic mass spectra are obtained.

Depending on the ionization method, CZE can be coupled to a mass spectrometer either directly (online) or indirectly (offline). In the latter mode of operation, $^{252}$Cf plasma desorption and matrix assisted laser desorption can be used. The online coupling of CZE with electrospray ionization mass spectrometry (ESI-MS) is more common and usually performed by electrospray ionization (ESI) or fast atom (ion) bombardment (FAB). Although online CZE/MS is the more common form of application, offline analysis has the advantage of allowing separation in non-volatile buffers, which are highly undesired in ESI.

It goes without saying that every analytical technique has its limitations and CZE/MS is no exception. One of the main limitations of this experimental arrangement is its relatively poor sample concentration/ion sensitivity. Approaches to reduce such limitations included online preconcentration, sample stacking, and the increasing use of time-of-flight (TOF) analysers which use ESI and TOF analysers with and without a quadrupole in between. The innovative feature of this class of instruments is their fast scanning, which allows the acquisition of a number of full spectra per second. Additionally, as all ions in each spectrum are sampled at the same moment in time, spectra are free of mass discrimination or peak skew typical of slow scanning systems that must scan over a narrow chromatographic/electrophoretic peaks.

Capillary electrochromatography (CEC) is another technique which is currently undergoing a rapid phase of advancement and development. This technique was revived by Jorgenson and Lukacs in 1981; these authors used 0.005 mol L$^{-1}$ phosphate buffer, 170 μm packed column and 30 kV separation voltage to separate 9-methylanthracene and perylene. This technique has recently become more diffuse because of a number of advances in both CZE instruments and detection techniques including electrospray mass spectrometry. However, on-column UV detection and in-column laser-induced fluorescence detection remain the most commonly used methods. Despite its high sensitivity, the latter method is subjected to interferences by buffer fluorescence. In MS detection, the column is commonly packed right up to the point where the sample is injected into the mass spectrometer. The combination of CEC with mass spectrometry provides reliable molecular weights and in many cases structural information, which makes it highly attractive for a wide range of applications. For more details on this topic, the reader is referred to recent extensive reviews, covering the methodology of CEC and its coupling to MS, by Colón et al. (1997) and Rentel et al. (1999). Interestingly, packed-CEC offers the possibility of higher sample capacity and the utilization of simpler mobile phases, which are more compatible with MS.

Experimental Aspects

One of the main advantages of CZE is that it requires simple instrumentation, which generally consists of
a high-voltage power supply, two buffer reservoirs, a capillary and a detector. Coupling of such instrumentation to a mass spectrometer requires the replacement of one of the buffer reservoirs with a suitable interface and some simple electronic circuit to accommodate the presence of an additional 3–5 kV required for the operation of the ion source.

Interfacing CZE to a mass spectrometer has been effected in a number of ways, yet all of them can be traced to two general configurations: liquid junction interface and coaxial sheath flow. The liquid junction interface, first described by Minard et al. in 1988, was used to couple a CZE to a continuous-flow FAB source. In such a configuration the CE capillary terminates in a 20 μm block that contains either the matrix solution for FAB ionization or the sheath solution for ES ionization. The inlet of the transfer capillary is aligned in close proximity to the cathode end of the CE capillary, an alignment considered critical, since the gap must be sufficiently wide to allow enough matrix or sheath fluid to maintain a stable ion beam. On the other hand, the same gap should be small enough to prevent analyte diffusion, upon exiting the CE capillary, which would otherwise result in peak broadening. The main disadvantage of this configuration is associated with the dead volume caused by a long transfer line which together with the mixing effects in the interface and the presence of the FAB matrix renders the separation efficiency of this interface poorer than that of the coaxial sheath flow. Liquid-junction interface has been redesigned to allow easier mounting and alignment of the CE and continuous-flow FAB capillaries. The same interface was further modified by Caprioli et al. in 1989, who used it for the analysis of synthetic mixtures of peptides and protein digests.

The first successful coupling of CE with MS was effected by Olivares et al. in 1887, wherein the cathode end of the CE capillary terminated within a stainless-steel capillary which completed the electrical circuit and established contact with the CE side. An improved version of this interface was developed by the same research group, where the metal contact at the CE terminus was replaced by a thin sheath of liquid flow. In comparison with the liquid-junction interface, the coaxial sheath–flow interface provided both better sensitivity and better resolution, yet this did not mean that the interface was trouble free. A number of difficulties can derive from the use of sheath liquid: ionic and neutral species within this liquid compete for protonation in the ESI process, thus lowering the overall sensitivity. The composition of the sheath liquid commonly includes a volatile organic acid (1% formic or acetic acid) in a mixed water–organic solvent, a composition which is different from that of the electrophoretic buffer. During the CE, sample ions and other species present in the buffer exit the capillary at the MS end, and, simultaneously, counterions from the liquid sheath enter the column and migrate toward the injection end. These moving ion boundaries can influence migration order, times and resolution. The same phenomenon has the advantage of allowing some analysis in the presence of difficult-to-spray electrolytes (such as phosphate- or borate-containing buffers).

**Figure 1** gives the up-to-date version of a commercial coaxial sheath–flow interface constructed and marketed by Micromass (Manchester, UK).

![Figure 1](image) The main components of a sheath–flow probe which can be used to couple CZE to a Q-TOF or to a single/triple quadrupole instruments. (Courtesy of Micromass, Manchester, UK.)
Representative Examples

CZE/MS in Peptide Analysis

One of the advantages of coupling CZE to ES mass spectrometry is the formation of multiply charged ions which allows simple analysers such as quadrupoles to measure the mass to charge ratios (m/z) of relatively large biomolecules. Indeed, the majority of CZE/MS applications have been in the field of biological and biochemical research. A number of reports have appeared on the characterization of synthetic mixtures of peptides and proteins, where the unusually high resolution of CZE permits the separation of sequences which may differ by a single amino acid residue. This advantage has been exploited in the use of CZE/ES–MS to examine a number of reaction mixtures of peptides obtained by solid-phase synthesis; two representative examples are considered here. A reaction mixture obtained by solid-phase synthesis of neuropeptide Y (NPY) analogue, [Leu$^{31}$, Pro$^{34}$]–NPY. This peptide has 36 amino acid residues, a relative molecular mass of 4222 and is known to play a major role in the central and peripheral nervous system. Online CZE/ES–MS analysis of this mixture resulted in a fairly complex UV and total ion current (TIC) electropherograms which, in addition to the target peptide, contained a number of side products. The use of mass spectrometry allowed reliable identification of all the components of the mixture, although some of the side products differed by a single amino acid residue in sequences containing over 30 residues. These measurements allowed unambiguous identification of the various components of the mixture which are summarized in Table 1.

A second and less complex reaction mixture associated with the same synthesis was also examined by the same technique which yielded the total ion current electropherogram in Figure 2(A). Deconvolution of the mass spectra in Figure 2 (panels 1–4) yielded the relative molecular masses 4222 (1), 2441 (2), 3577 (3) and 3789 (4). The first molecular mass coincides with the desired peptide, while the other three masses are associated with a number of incomplete sequences which are summarized in Table 2 which clearly shows an excellent agreement between the calculated and measured masses, fully based on CZE/MS measurements. Given the complex procedure associated with solid-phase synthesis of peptides, it is evident that the use of online CZE/MS is an indispensable analytical tool for the initial characterization of the product and for providing a reasonable indication on the yield of synthesis.

CZE/MS can also give reliable information on unexpected processes in the course of solid-phase synthesis of peptides. To underline this statement, two cases are considered. A series of newly synthesized peptides investigated, corresponding to portions of the extracellular domain of human granulocyte-macrophage colony-stimulating factor receptor α-subunit. A solution containing 3 mg mL$^{-1}$ of the peptide [PRAKHSVIRIADVRILN], $M_r = 2084$, was examined by full-scan CZE/MS, which yielded UV and TIC electropherograms, each of which contained three peaks of almost equal relative heights. The associated ES mass spectra revealed the presence of the desired peptide together with its acetylated version in one of the three TIC peaks. It is fair to say that without the use of online MS detection, the identification of the latter component would have been very unlikely. A second case refers to online CZE/MS of an NPY analogue which exhibited a TIC electropherogram containing seven peaks, two of which yielded $M_r = 4222$ which implied the presence of two different configurations of the same sequence within the same crude of synthesis. This deduction was found in accord with existing literature describing undesirable side products observed in solid-phase synthesis of peptides and small proteins. One such side product can be invoked by the formation of succinimide of the Asp residue (β-aspartyl peptide) which, in the present case, has the same $M_r$ as the

<table>
<thead>
<tr>
<th>Peptide chain</th>
<th>$M_r$ calculated</th>
<th>$M_r$ measured</th>
<th>Proposed sequence</th>
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</thead>
<tbody>
<tr>
<td>Ac[Leu$^{31}$, Pro$^{34}$]–NPY</td>
<td>4222</td>
<td>4222 ± 1</td>
<td>Ac-YPSKPDPGEPADAEDLARYYSALRHYINLTRLPRY-NH$_2$</td>
</tr>
<tr>
<td>H-12-36-NH$_2$</td>
<td>3022</td>
<td>3022 ± 1</td>
<td>H-AEDAEDLARYYSALRHYINLTRLPRY-NH$_2$</td>
</tr>
<tr>
<td>Ac[Leu$^{31}$, Pro$^{34}$]–NPY</td>
<td>4222</td>
<td>4222 ± 1</td>
<td>Ac-YPSKPDPGEPADAEDLARYYSALRHYINLTRLPRY-NH$_2$</td>
</tr>
<tr>
<td>Ac(5–36)</td>
<td>3789</td>
<td>3789 ± 1</td>
<td>Ac-PDPGEPADAEDLARYYSALRHYINLTRLPRY-NH$_2$</td>
</tr>
<tr>
<td>Ac(4–36)</td>
<td>3917</td>
<td>3917 ± 1</td>
<td>Ac-KDPGEPADAEDLARYYSALRHYINLTRLPRY-NH$_2$</td>
</tr>
<tr>
<td>Ac(3–36)</td>
<td>4004</td>
<td>4004 ± 1</td>
<td>Ac-SKPDPGEPADAEDLARYYSALRHYINLTRLPRY-NH$_2$</td>
</tr>
<tr>
<td>Ac(20–36)</td>
<td>2440</td>
<td>2441 ± 1</td>
<td>Ac-YYSALRHYINLTRLPRY-NH$_2$</td>
</tr>
<tr>
<td>Ac(7–36)</td>
<td>3577</td>
<td>3577 ± 1</td>
<td>Ac-NPGEPADAEDLARYYSALRHYINLTRLPRY-NH$_2$</td>
</tr>
<tr>
<td>Ac(5–36)</td>
<td>3789</td>
<td>3789 ± 1</td>
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NPY analogue. The mechanism responsible for such reaction is depicted in Figure 3.

**Forensic Application of CZE/MS**

Most drugs of forensic interest are commonly analysed by GC/MS or extraction followed by IR analysis. Separation of benzodiazepines and ergot alkaloids by GC is difficult and differences in the IR spectra are small. On the other hand, the analysis of these compounds by CE/MS is characterized by short analysis times and specific MS information. Forensic drug chemists, investigating drugs of abuse, also find the analysis of drugs such as LSD challenging because of microgram quantity dosage and the fact that GC/MS analysis is rather demanding owing to column adsorption and thermal lability. This is also true for psilocybin, the psychoactive agent in certain

Figure 2  Upper frame: total ion current (TIC) electropherogram of a crude of synthesis associated with [Leu<sup>31</sup>,Pro<sup>34</sup>]-NPY. Lower frames: associated positive ES mass spectra of the TIC peaks: (1) $t_M = 59.61$ min; (2) $t_M = 64.41$ min; (3) $t_M = 68.30$ min; and (4) $t_M = 70.02$ min.
Table 2  Proposed peptide sequences, corresponding to relative molecular masses ($M_r$) obtained from the ES mass spectra associated with the components arising from the synthesis of [Leu$^{31}$,Pro$^{34}$]-NPY

<table>
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<td>2440</td>
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<td>Ac(5--36)</td>
<td>3789</td>
<td>3789 ± 1</td>
<td>Ac--PDNPGEDAPAEDLARYYSALRHYINLLTRPRY-NH$_2$</td>
</tr>
</tbody>
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Figure 3  Representation of succinimide formation using partial sequence containing Asp$^6$ and two adjacent amino acids, Asn$^7$ and Pro$^9$ taken from the sequence of [Leu$^{31}$,Pro$^{34}$]-NPY. (Reproduced with permission of John Wiley & Sons Ltd.)
mushrooms, owing to the presence of the highly labile phosphate moiety. The absence of complex preparative extractions for CE analysis of both drugs allows their separation without causing undesired decomposition.

The fact that many forensic drugs have closely related isomeric structures renders CE/MS a powerful tool for their separation and identification. For instance, phenethylamines are historically a difficult group to analyse due to the large number of isomers including both D- and L-optical isomers. Twelve optical isomers of ephedrine, pseudoephedrine, norpseudoephedrine, amphetamine and methamphetamine have been separated in under 30 min using CE. It is worth noting that this class of compounds tends to give strong \([\text{M} + \text{H}]\) signals in ES ionization which makes them ideal for CZE/MS analysis.

A separation of 18 common drugs of abuse was accomplished in less than 40 min using CE buffered with phosphate/borate, with sodium dodecyl sulfate (SDS) as a micellar phase and acetonitrile as an organic modifier. The main advantage of this method over other screening techniques is that acidic, basic and neutral drugs can be screened through a single analytical method, where extraction procedures are unnecessary.

Clandestine manufacturers of illegal drugs pose additional challenges to the forensic scientist, since samples submitted for analysis tend to contain complex mixtures of chemicals, including thermally reactive and labile components. CZE with and without MS detection can be a powerful tool for such type of analysis. For example, the frequently encountered methamphetamine can be analysed by CE allowing the identification of its isomeric composition which can be eventually used to construct evidence concerning its synthetic pathway and the source of the sample.

An additional problem with samples from clandestine laboratories is the presence of unreacted precursors and adulterants that may interfere with the analysis of the target compound(s). For instance, under typical GC/MS conditions, certain mixtures of chemicals associated with methamphetamine will derivatize the illegal drug to a compound that is not controlled, resulting in an inconclusive analysis. Using a number of CE methods, such interference can be easily avoided.

CZE/MS is also highly suitable for the analysis of biological samples which may contain traces of illegal drugs or poisons in a complex matrix. Extensive reviews cite over sixty references on the use of CE for the analysis of drugs in biological samples including drugs of abuse in urine, cocaine and morphine in hair and barbiturates in serum. Other examples include the chiral separation of racemethorphan and racemorphan in urine using a cyclodextrin/SDS/propanol buffer and the micellar CE separation of nitrazepam and its metabolites in urine. An extensive review is available on the use of CZE coupled to tandem mass spectrometry to identify a variety of drugs and metabolites. All these reviews are listed in the Further Reading.

**Forensic Analysis of Inorganic Explosives**

Capillary electrophoresis is also widely used in the analysis of inorganic ions in criminal cases, material such as black powder, flash powder, ammonium nitrate and home-made explosive mixtures. The inorganic anions resulting from an explosive reaction of such materials are among the most important evidence used to determine the nature of an inorganic explosive. For many years, the most powerful tool in these investigations was ion chromatography (IC). The introduction of CE for anion analysis provided a simpler, faster and slightly more sensitive technique for performing such analysis. It is interesting to note that CE separations are based on differences in charge-to-mass ratios of the solvated ions, while IC separations are the result of complex interactions between the ions and the stationary phase. As a result, the migration order is quite different in the two methods and a nearly orthogonal relationship exists between the relative retention times.

The reliable analysis of certain alkyl-substituted organophosphorus acids, which are the primary hydrolysis products of neurotoxic agents, has become very important in the last few years owing to the likelihood of an international agreement that will forbid the development, production and stockpiling of chemical warfare agents and weapons. CZE coupled to ion spray mass spectrometry was applied in the negative ion mode to investigate five organophosphonic acids, which are the primary hydrolysis products of neurotoxic agents. The MS spectra exhibited a very abundant \([\text{M} – \text{H}]^-\) signal with minimal fragmentation. The authors reported sensitivity in the range 10–30 pg using the single-ion recording (SIR) mode.

**Conclusions**

The examples cited in this work have to be considered as only a part of the capabilities of CE with and without MS coupling. The literature cites varied areas of application of such powerful methodology. As new applications of CE continue to appear, the
advantages and importance of CE in conjunction with mass spectrometry have also become appreciated. Analytical chemists are faced with the challenge of increasing sample complexity and decreasing sample quantities. Because of the complexity observed with most biological mixtures, there continues to be a need for the development of a highly efficient separation technique in conjunction with a sensitive and specific detector. The low quantities of analytes often available require nanoseparation techniques. The mass spectrometer is a selective and broadly applicable detector for analytical separations. It can provide information regarding the structure of unknown components present in a sample mixture with high specificity and sensitivity. The coupling of CE with MS combines the extremely high-resolving power and structural information in one system. Like any other separation technique such as GC–MS and LC–MS, the principal advantage of CE–MS is that analytes are identified by both their differential separation and their molecular masses and/or fragmentation patterns. An analytical separation that precedes MS analysis is often necessary to assure correct interpretation of the mass spectral data.

Fast, high-efficiency separation techniques are becoming ever more important in the race to discover new drugs. The potential complexity of libraries produced by automated parallel synthesis, combinatorial and genetically manipulated natural product chemistries are driving many developments in separation sciences.

CE, CEC and nano-LC are all potential candidates for such analyses and each has a requirement for a fast, sensitive detection system.

**Further Reading**


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**Capillary Electrophoresis–Nuclear Magnetic Resonance**

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Miniaturization is an important current trend in separation science and the development of capillary electrophoresis (CE), capillary HPLC (cHPLC) and capillary electrophoretic chromatography (CEC) are milestones in this respect. The electrophoretic techniques especially can achieve rapid and efficient separations using only very small volumes and they have become research tools with widespread applications. The advantages of this miniaturization are obvious: less sample is